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MICROPROPAGATION

Effect of nutrient media on axillary shoot proliferation and preconditioning for adventitious shoot regeneration of pears

Richard L. Bell · Chinnathambi Srinivasan · Delores Lomberk

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Abstract The influence of the nutrient composition of plant tissue culture media on axillary shoot proliferation and their preconditioning effect on subsequent adventitious shoot regeneration from pear leaves was investigated. The goal was to improve both micropropagation and regeneration of 'Bartlett' and 'Beurre Bosc' pear cultivars. Driver–Kuniyuki walnut (DKW) and Quoirin and Lepoivre (QL) nutrient media were found to be superior to Murashige and Skoog (MS) and Woody Plant Medium (WPM) for axillary shoot proliferation. Shoots on WPM exhibited some chlorosis. Axillary shoot culture on DKW would be preferred to that on QL due to the production of excessively short thin shoots on the latter medium. DKW also was superior to QL and MS for production of young expanding leaves for use as explants in adventitious regeneration. Leaf explants derived from shoot proliferation cultures grown on DKW or QL media produced more adventitious shoots than leaf explants from MS.

Keywords 'Bartlett' · 'Beurre Bosc' · Biotechnology · Plant tissue culture · *Pyrus communis*

R. L. Bell (✉) · C. Srinivasan
U. S. Department of Agriculture, Agricultural Research Service,
Appalachian Fruit Research Station,
2217 Wiltshire Road,
Kearneysville, WV 25430-2771, USA
e-mail: richard.bell@ars.usda.gov

D. Lomberk
U. S. Department of Agriculture, Agricultural Research Service,
Epcot Science,
2013 North Avenue of the Stars,
Lake Buena Vista, FL 32830, USA

Introduction

Micropropagation methods are used for rapid clonal multiplication of pear and other woody plant species. They are also general prerequisites to exploiting somaclonal variation and induced mutations and for the development of transgenic plants. Protocols for micropropagation of pear have been published, beginning in the late 1970s, for over 20 genotypes, including the major *Pyrus communis* L. cultivars and genotypes of four other species. These studies were reviewed by Chevreau et al. (1992) and by Bell and Reed (2002). Empirical studies to determine optimum cultivar-specific protocols have been conducted for a few, but not all, of the major cultivars of several *Pyrus* species.

While many studies have concentrated on the influence of plant growth regulators, the influence of the nutrient medium has received less attention. Most studies have used Murashige and Skoog (MS; Murashige and Skoog 1962) medium without modification, but a few have reduced the overall ion concentration or modified the nitrogen concentration or nitrogen sources (Chevreau et al. 1992; Bell and Reed 2002). Only a few of the studies made comparisons among several nutrient media. Nedelcheva (1986) found that shoot proliferation of 'Bartlett' was the greatest on a medium devised by Quoirin and Lepoivre (1977; QL), in comparisons with MS medium. In contrast, Baviera et al. (1989) obtained better shoot proliferation of 'Conference' on MS than QL. Wang (1991) observed a higher degree of multiple shoot formation of the *P. communis* L. rootstock BP10030 on Woody Plant Medium (WPM; Lloyd and McCown 1981) and QL than MS in a double-phase culture system consisting of a liquid medium overlaid on semisolid medium. Yeo and Reed (1995) found that the nutrient medium of Cheng (1979) was better for shoot multiplication than WPM for a genetically diverse group of root-

stocks, 'OH × F 230' (*P. communis* L.), 'OPR 260' (*Pyrus betulifolia* Bunge), and 'OPR 157' (*Pyrus calleryana* Decne.). Likewise, Thakur and Kanwar (2008) reported that WPM resulted in enhanced axillary shoot proliferation of *Pyrus pyrifolia* when compared to MS and various modifications, but Banno et al. (1989) found that it was true for only two of six cultivars.

The major differences in macronutrients among these media are in ammonium and nitrate ion concentrations and total ion concentration. Full-strength MS is high in ammonium (20.6 mM) and nitrate ions (39.4 mM), while QL is a low ammonium medium (5 mM). WPM contains low concentrations of both ammonium (5 mM) and nitrate (9.7 mM) ions. In addition, QL uses calcium nitrate as a nitrogen source. A medium originally developed for walnut (Driver and Kuniyuki 1984), designated DKW, also has lower ammonium ion content (17.7 mM) than MS and contains calcium nitrate instead of potassium nitrate. The efficacy of DKW for axillary shoot proliferation of pear has not been assessed.

Adventitious shoot regeneration can be affected by various preconditioning factors, including type of cytokinin in the shoot proliferation medium (Swartz et al. 1990; Chevreau and Leblay 1993; Bell 1995) and use of rooted shoots (James et al. 1988), presumably through the influence of size or endogenous plant growth regulators on explant quality (Mullins 1967). Young expanding apical leaves of pear regenerate adventitious shoots at a greater frequency than older more basal leaves (Chevreau and Leblay 1993) and are easier to handle than the youngest unfurled apical leaves. The influence of the nutrient composition of shoot proliferation medium on subsequent adventitious shoot regeneration from leaf explants has not been addressed.

We report the results of a preliminary experiment which addressed the preconditioning effect of shoot proliferation medium on adventitious shoot organogenesis. A second experiment was conducted to determine whether the nutrient medium influenced axillary shoot proliferation, the number of young expanding leaves usable as explants for regeneration experiments, and to confirm the subsequent preconditioning effect on the frequency of adventitious shoot organogenesis from leaves. The objective was to improve the efficiency of production of leaf explants suitable for transformation and regeneration experiments involving 'Bartlett' and 'Beurre Bosc'.

Materials and Methods

Plant material. Axillary shoot cultures of the European pear cultivars, 'Bartlett' and 'Beurre Bosc' (*P. communis* L.), were established from *in vitro* cultures of the virus-free clones CPYR 38.001 and CPYR 1165.001 obtained from the US Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository, Corvallis, OR. The cultures were maintained in Magenta™ GA-7 tissue culture vessels (Sigma Chemical Co., St. Louis, MO) on 50 ml of shoot proliferation medium consisting of MS macro- and micronutrients (Murashige and Skoog 1962), MS organics, 5 μM 6-benzylaminopurine, 0.5 μM indole butyric acid (IBA), 0.6 μM gibberellic acid-3 (GA_3), 30 g l^{-1} sucrose, and 6 g l^{-1} high gel strength tissue culture agar (A9799, Sigma Chemical Co.), with pH adjusted to 5.8 with 1 M KOH. Shoots, approximately 1 cm in length and possessing two to three axillary buds, were excised and transferred to fresh medium approximately every 4 wk for 4 mo.

Regeneration from leaves derived from cultures grown on three media (experiment 1). Shoot explants, consisting of single shoot sections trimmed to three nodes, were transferred from the MS-based source culture to fresh MS medium and two additional media, DKW and WPM, which differed in the macro- and micronutrients. MS organics were used with MS and WPM media. Aliquots of 50 ml of the media were dispensed into Magenta™ GA-7 vessels (Sigma Chemical Co.). Nine leafless shoots which had been trimmed to three nodes each were transferred to each of ten vessels per medium and placed into the medium surface in a horizontal orientation. The cultures were grown in a tissue culture room at 25°C, with ~45 μmol m^{-2} s^{-1} illumination provided by cool white fluorescent bulbs, and 16-h light for a period of 4 wk. The two fully expanded apical leaves were excised from shoots on each medium at weekly intervals to determine their potential for adventitious shoot regeneration. Each leaf was wounded by making three cuts transversely across the midrib and explanted adaxial side down onto a shoot regeneration medium containing the macro- and micronutrients, organics, and sucrose according to Chevreau and Leblay (1993), 10 μM thidiazuron, 5 μM IBA, 3.5 g l^{-1} gellan gum (Phytagel™, Sigma Chemical Co.) at a pH of 5.8. Ten leaves were placed into each of six Petri dishes (100×25 mm), each containing 20 ml of medium, and incubated in the dark in the tissue culture room. After 4 and 8 wk, the explants were transferred to auxin-free fresh medium and incubated in the light. The number of adventitious shoots which developed on each explant was recorded at the end of each transfer period. Data on the number of adventitious shoots per explant for each medium × cultivar combination were tested for normality by Shapiro–Wilk *W* statistic (SAS Institute Inc. 1989). Because all distributions differed significantly from normal and there were some explants with no adventitious shoots, the number of regenerant shoots was transformed by the square root of (n+0.5). Arcsin transformations were performed on the proportions of regenerating explants per

plate, with Bartlett's adjustment for 0 and 100% (Steel and Torrie 1960). The transformed proportions were subjected to a two-way factorial analysis of variance using SAS MIXED (Littell et al. 1996), with medium and cultivar considered to be fixed effects and plates as a random nested effect. Least square means were computed, and difference among cultivars was tested by *t* test, while differences among media were tested by Tukey's HSD.

Shoot proliferation and preconditioning of shoots on three media (experiment 2). Temperature and illumination conditions were the same as those of experiment 1. Explants consisted of one to three attached shoots from MS-based source cultures. Each shoot was trimmed back to approximately three nodes and transferred to a medium which differed only in that it was based upon DKW nutrients. Explants were placed into the medium surface in a 45° inclined orientation. The cultures were grown for 1 mo on this medium to increase the number of explants before transfer to experimental treatments. The experimental treatments consisted of three media which differed in their macro- and micronutrients and organics. The nutrient media were MS, DKW, and QL. MS organics were used with MS and QL media. The other components were those listed above for experiment 1. The cultures were grown on each medium for 4 wk (transfer generation 1) to allow the cultures to acclimate to the new media. Shoot explants were then excised and trimmed as above. The number of nodes of each explant and the fresh and dry weights of one random explant from each of ten vessels were recorded prior to explants being transferred to fresh medium. After 4 wk, data on the number of new shoots per explant, total number of expanding leaves per explant, and the fresh and dry weights of one random explant from each of ten vessels were recorded. The difference between initial and final dry weights was calculated. Explants were excised and transferred to the same fresh media two additional times and identical data collected to determine repeatability through the third shoot proliferation transfer generation.

The dry weight gain data were analyzed as a fixed-effects factorial model, with initial dry weight as a covariate, using SAS PROC GLM, with type III sums of squares. The numbers of new shoots were analyzed according to a factorial model, with vessel as a nested random effect and initial number of nodes as a covariate, using SAS PROC MIXED (Littell et al. 1996). Fixed main effects in all analyses were cultivar, medium, and transfer generation. Least square means were calculated and differences among means for significant effects were tested by *t* test or Tukey's honestly significant differences (HSD), as appropriate for single or multiple comparisons, respectively.

Starting at 2 wk after transfer and continuing weekly to 6 wk, the numbers of young expanding leaves apparently usable as explants for regeneration were counted, and the data analyzed according to a fixed-effects factorial model using SAS PROC GLM, with initial number of nodes as a covariate. Forty leaves, generally the two fully expanded apical leaves, were excised from shoots from two vessels of each medium × cultivar combination to determine their potential for adventitious regeneration. Each leaf was wounded by making three cuts transversely across the midrib and placed abaxial side down onto the same shoot regeneration medium used for experiment 1, except that the IBA concentration was 12.5 μM. The high IBA concentration is that which we have employed in transformation experiments. Ten leaves were placed into each of four Petri dishes (100×25 mm), each containing 20 ml of medium, and incubated in the dark in the tissue culture room. After 4 wk, the explants were transferred to fresh, but auxin-free medium, and returned to the dark. After an additional 4 wk, the explants were transferred to fresh auxin-free media and incubated in the light for 4 wk. The number of adventitious shoots per explant was recorded at the end of each transfer period, but only the final data is presented here. Most of the 6-wk-old 'Bartlett' explants from DKW in the second transfer became contaminated, perhaps due to growth of internal microbial contaminants. Therefore, only data on 2 through 5-wk-old explants were analyzed. Data on the proportion of regenerating explants and the total number of regenerant shoots were analyzed according to a fixed effects model using SAS PROC GLM (SAS Institute, Inc. 1989). Explant age was included in the model as a quantitative effect. Least square means of qualitative fixed main effects (*i.e.*, cultivar, medium, and transfer generation) and interaction means were calculated and differences among means for significant effects were tested by Tukey's HSD.

Results

Regeneration from leaves derived from cultures grown on three media (experiment 1). The analysis of variance indicated that the overall mean percentage regenerating explants for the main effects of media and cultivar were not different, and the medium × cultivar interaction was also not significant. Differences in the mean number of regenerant shoots per explant due to medium were highly significant, while the effects of cultivar and medium × cultivar interaction were not significant. The use of DKW or WPM as the shoot proliferation medium resulted in significantly greater percentages of regenerating explants of 'Beurre Bosc', but not of 'Bartlett'. When averaged over both cultivars, the use of DKW for the explant source resulted in significantly greater numbers of regenerant shoots per explant than WPM and MS (Table 1). When cultivars were considered separately, media did not differ

Table 1. Preconditioning effects of shoot proliferation media on adventitious shoot regeneration of 'Bartlett' and 'Beurre Bosc' pear leaves

	% Regenerating explants			Mean no. regenerant shoots/explant		
	Medium Bartlett	Beurre Bosc	Bartlett	Media mean	Beurre Bosc	Media mean
Experiment 1						
DKW	56.7 a[a]	66.7 a	61.7 a[a]	0.97 a	0.92 a	0.95 a
WPM	52.0 a	54.0 a	53.0 a	0.64 a	0.68 a	0.66 b
MS	48.3 a	35.0 b	41.7 a	0.72 a	0.40 b	0.56 b
Cultivar mean	52.3 a[b]	51.9 a		0.78 a	0.67 a	
Experiment 2						
DKW	45.7 a	46.7 a	46.2 a	0.64 a	0.74 a	0.69 a
QL	42.8 a	42.2 a	42.5 a	0.57 ab	0.66 b	0.62 ab
MS	34.6 a	41.7 a	38.2 a	0.40 b	0.60 b	0.50 b
Cultivar mean	41.0 a	43.5 a		0.56 b	0.67a	

[a] Mean separation of media means and of cultivar by medium means in the same *column* were tested by Tukey's HSD, $P \leq 0.05$

[b] Mean separation of cultivar means within *column* were tested by LSD, $P \leq 0.05$.

for 'Bartlett', and DKW and WPM were superior to MS for 'Beurre Bosc'. Overall, 'Bartlett' and 'Beurre Bosc' did not differ significantly from each other in any measure of regeneration. Shoots on WPM exhibited some chlorosis.

Shoot proliferation and preconditioning of shoots on three media (experiment 2). The greatest shoot proliferation of both 'Bartlett' and 'Beurre Bosc' was obtained on QL, followed by DKW and MS medium (Table 2). Over all three media, the two cultivars did not differ significantly in the number of new axillary shoots which proliferated. The two cultivars did not differ significantly in their response to the three media. However, the shoot clumps which developed on QL were characterized by numerous short thin shoots, with small narrow leaves, especially during the first transfer generation. The leaves were difficult to excise, score, and otherwise handle in subsequent regeneration experiments. Explants on MS were observed to develop more shoot tip necrosis than those cultured on DKW or QL.

The analysis of covariance indicated that initial dry and fresh weights did not significantly affect weight gains during culture (Pr > F=0.22 and 0.12, respectively). The mean dry weight gain of 'Beurre Bosc' explants was

Table 2. Effect of three nutrient media on axillary shoot proliferation, dry weight gain, and leaf production of 'Bartlett' and 'Beurre Bosc' pear

Effect and treatment	Mean shoots/initial no. nodes	Mean dry weight gain (mg)	Mean usable leaves/initial no. nodes
Cultivar			
Bartlett	2.23 a[a]	60.1 b	1.45 a
Beurre Bosc	1.93 a	75.5 a	1.29 b
Medium			
QL	2.85 a[b]	71.6 a	1.38 b
DKW	1.94 b	77.8 a	1.61 a
MS	1.45 c	54.0 b	1.11 c
Cultivar × medium			
Bartlett-QL	3.14 a	54.8 b[b]	1.55 a
Bartlett-DKW	1.94 b	81.6 a	1.53 a
Bartlett-MS	1.61 b	43.9 b	1.26 b
Beurre Bosc-QL	2.55 a	88.4 a[b]	1.20 b
Beurre Bosc-DKW	1.93 b	73.9 b	1.70 a
Beurre Bosc-MS	1.30 c	64.1 b	0.97 b
Transfer			
Second	2.52 a	58.7 b	1.73 a
Third	1.64 b	76.9 a	1.00 b
Transfer × medium			
Second—QL	3.12 a	62.2 a	1.76 a
Second—DKW	2.63 a	63.1 a	2.16 a
Second—MS	1.83 b	50.8 a	1.26 b
Third—QL	2.58 a	82.8 a	0.99 a
Third—DKW	1.25 b	89.8 a	1.07 a
Third—MS	1.08 b	57.9 b	0.97 a

[a] Mean separation of cultivar and transfer means within *column* tested by LSD, $P \leq 0.05$

[b] Mean separation of medium, medium within cultivar, and medium within transfer means in same *column* were tested by Tukey's HSD, $P \leq 0.05$

significantly greater than that of 'Bartlett' (Table 2). Explants grown on DKW and QL media gained approximately 40% more dry weight than explants grown on MS. The medium by cultivar interaction was significant (Pr > F=0.04). Explants of 'Beurre Bosc' gained significantly more dry weight on QL than on DKW and MS, while explants of 'Bartlett' performed significantly better on DKW. Explants grown during the third transfer gained 25% more dry weight than those of the second transfer generation (77 mg *vs.* 59 mg, respectively).

The mean number of usable leaves per initial number of explant nodes was significantly greater in the second generation than the third (1.73 *vs.* 1.00, respectively). 'Bartlett' produced significantly more usable leaves than 'Beurre Bosc' (1.45 *vs.* 1.29, respectively), and shoots on DKW medium produced the largest number of usable leaves (Table 2). Although there was some deviation from a linear increase with age in QL cultures, the number of usable leaves produced on each medium increased with age, with approximately twice as many leaves available for regeneration in 6-wk-old cultures than 2-wk-old cultures (1.9 *vs.* 0.9, respectively; Pr > F=0.0001). DKW produced significantly more usable 'Beurre Bosc' leaves; DKW and QL produced significantly more usable 'Bartlett' leaves than MS medium. After the second transfer, cultures on DKW and QL both produced more usable leaves than those on MS, but after the third transfer, the number of usable leaves from DKW and QL decreased, such that there were no significant differences among the three media. Therefore, the efficiency of leaf production was not consistent between transfers.

There were no significant differences in the percentage of regenerating explants for both cultivars and overall due to proliferation media (Table 1; experiment 2). When averaged over both cultivars and for 'Bartlett', leaf explants from DKW medium produced significantly more regenerant shoots than those from MS. DKW-derived 'Beurre Bosc' explants were superior to those from both QL and MS. Explants from 'Beurre Bosc' produced approximately 10% more regenerants than 'Bartlett'. The generally lower percentage of regenerating explants and mean numbers of regenerant shoots between experiments 1 and 2, particularly for DKW-derived explants, may be due to differences in the regeneration protocols. The higher concentration of IBA in experiment 2 may have adversely affected regeneration.

Overall, the number of regenerating shoots per explant increased significantly from the first (0.55) to the third transfer generation (0.67), but explants from the different media responded differently, with MS explants significantly improving (0.38 to 0.68), while the trends for DKW and QL explants were nonsignificant. The explant age by medium interaction was significant (Pr > F=0.0001), but no consistent trend was evident for any medium (data not presented). The transfer generation by explant age interaction was also significant (Pr > F=0.007), but again the variations did not fit a consistent pattern. No consistent patterns of practical importance could be discerned to explain the significant three-way interactions. The mean proportion of regenerating explants per plate varied significantly only among transfer generations, increasing from 0.38 in the first transfer to 0.47 in the third (Pr > F=0.048).

Table 3. Ion concentrations (mM) of shoot proliferation media

	Medium			
	DKW	MS	QL	WPM
Macronutrients (mM)				
NH_4^+	17.7	20.6	5.0	5.0
NO_3^-	34.4	39.4	33.0	9.7
PO_4^-	2.0	1.3	2.0	1.3
SO_4^-	12.3	1.7	1.6	7.2
K^+	20.0	20.0	19.8	12.6
Mg^+	3.0	1.5	1.5	1.5
Ca^+	9.3	3.0	5.1	3.0
Cl^-	2.0	6.0	–	1.3
Fe^{++}	0.1	0.1	0.1	0.1
Na^+	0.3	0.2	0.2	0.2
Micronutrients (μM)				
B^{+++}	77.6	100.0	100.0	100.0
Co^{++}	–	0.1	0.1	–
Cu^{++}	1.0	0.1	0.1	1.0
I^-	–	5.0	0.5	–
Mn^{++}	198.0	100.0	44.8	132.0
MoO_4^-	1.6	1.0	1.0	1.0
Ni^+	0.02	–	–	–
Zn^{++}	57.2	29.9	29.9	29.9
Summary values for inorganic components				
Total N	52.1	60.0	38.0	14.8
NO_3^-/NH_4^+	1.9	1.9	6.6	2.0
Total				
Molarity	101.4	94.0	68.5	42.2
Vitamins and organics (μM)				
Glycine	26.6	25.0	–	–
Myo-inositol	555	555	–	–
Nicotinic acid	8.1	4.1	–	–
Pyridoxine HCl	–	2.4	–	–
Thiamine HCl	5.9	0.3	–	–

Macro- and micronutrient concentrations are based upon conversion from milligrams per liter amounts according to commercial formulations (Sigma-Aldrich 2008). Vitamin and organics are derived from Driver and Kuniyuki (1984) and Murashige and Skoog (1962)

Discussion

Murashige and Skoog nutrient medium has been most commonly used for axillary shoot proliferation of pear (Chevreau et al. 1992; Bell and Reed 2002), for the purposes of micropropagation and as a source of leaf explants for transformation and regeneration experiments. The present research compared four nutrient media which have been used by various authors for tissue culture of pear and other woody plant species. The results presented herein indicate that DKW and QL media were superior to MS for axillary shoot proliferation of 'Bartlett' and 'Beurre Bosc' pear cultivars. The differences among these media cannot be explained solely on the basis of total ionic strength, a high level of which can be inhibitory to *in vitro* growth of woody plant species (McCown and Sellmer 1987), since although QL has a total ion concentration 72% of MS, DKW is 8% higher than MS. The observed responses maybe due to the high calcium contents of DKW and QL or to the high chloride ion concentration of MS (Table 3). In addition, DKW is the only medium that contains Ni^{+}, although at an extremely low concentration, and contains nearly twice the concentration of Zn^{++} of the other three media. DKW organics and vitamins differed from those of MS, with no pyridoxine HCI, twice the nicotinic acid, and 20 times the thiamine HCl. This also may have contributed to the positive preconditioning effect of DKW media

Wang (1991) found also that QL was superior to MS for proliferation of the rootstock clone BP10030, but found, in contrast to our results, that WPM resulted in the greatest proliferation. The difference in results may be due to differences in the cultivars, or influenced by Wang's use of a double phase culture system. Our observations indicated that axillary shoot culture on DKW might be preferred to that on QL due to the production, at least initially, of excessively short thin shoots and narrow leaves on QL medium. DKW also was superior to QL and MS for production of quality leaf explants for adventitious regeneration. Leaf explants derived from DKW and QL produced more adventitious shoots than leaf explants from MS and WPM. Chlorosis of WPM cultures, due perhaps to the low nitrogen content (Table 3), may have adversely affected shoot regeneration from WPM-derived explants.

The relative effectiveness of the shoot proliferation media in production of leaves usable as explants for regeneration was the same as the experiment 2 results for the mean number of regenerants. DKW as the nutrient medium is recommended for micropropagation of 'Bartlett' and 'Beurre Bosc', and QL appears equally effective for 'Bartlett', especially when explants are to be used in subsequent transformation and regeneration experiments.

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